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Proteins Encoded by the *cag* Pathogenicity Island of *Helicobacter pylori* Are Required for NF-κB Activation

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***Helicobacter pylori* is the etiological agent in the development of chronic gastritis, duodenal ulceration, and gastric adenocarcinoma. The difference in virulence between individual strains is reflected in their ability to induce interleukin-8 (IL-8) secretion from gastric epithelial cells. It has been shown that virulence is associated with the presence of a bacterial gene cluster (a pathogenicity island). We have recently demonstrated that *H. pylori*-mediated IL-8 secretion requires activation of the transcription factor NF-κB. Here, we show that NF-κB induction requires six membrane proteins encoded within the pathogenicity island.**

Helicobacter pylori infection can cause a wide variety of diseases in humans. While most individuals develop only superficial gastritis, in a small proportion of individuals infection progresses to duodenal ulceration and gastric adenocarcinoma (4, 17, 18). This variability in the clinical manifestations of *H. pylori* infection is potentially due to differences in the virulence of individual *Helicobacter* strains. Until recently, the presence of a cytotoxin-associated antigen (*cagA*) was the best predictor of strain virulence. While *cagA* is present in 50 to 60% of *H. pylori* isolates from patients with gastritis, it is found in 88 to 100% of strains from patients with duodenal ulceration (7). Because of the strong inflammatory response to *Helicobacter* infections, the role of inflammatory cytokines was investigated. It was shown that mucosal biopsies from patients with *H. pylori* infections contain significantly elevated levels of interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), and IL-8 compared to those in specimens from uninfected individuals (9, 10, 12, 16). Moreover, among those infected, patients with active gastritis show higher levels of TNF-α and IL-8 than do patients with chronic gastritis (9, 12).

Exposure of gastric epithelial cell lines to *H. pylori* induces the secretion of IL-8 (8, 23). This model system was used to test various *H. pylori* strains for their ability to stimulate cytokine production. While *cagA*$^+$ strains induce significantly higher IL-8 levels than do *cagA*-negative strains (8, 14, 21), it was recently shown that isogenic *cagA* mutants elicit IL-8 to the same degree as does the wild-type parent strain (11, 23). Therefore, although *cagA* is a marker of enhanced pathogenicity, it is not the molecular mediator of the inflammatory response. It was shown last year that the *cagA* gene resides within a pathogenicity island, a DNA segment which contains over 40 genes encoding bacterial virulence factors (6). Using isogenic mutants, Censini et al. (6) demonstrated that several genes located within the pathogenicity island are required for the ability of *Helicobacter* to elicit IL-8 secretion from gastric epithelial cells. Therefore, the presence of a pathogenicity island is associated with increased virulence of a *Helicobacter* strain.

Genes encoding IL-8, IL-1β, IL-6, and TNF-α are targets for the human transcription factor NF-κB (2). This protein plays an integral role in regulating the human immune response. It is present in an inactive, cytoplasmic form in almost all cell types (1). NF-κB is activated upon stimulation by a large variety of pathogenic agents (2). Activation occurs via phosphorylation, ubiquitinilation, and proteolytic degradation of IκB, the inhibitory subunit (3, 5, 13, 24–26). The released NF-κB dimer rapidly translocates to the nucleus, where it activates transcription of target genes including those encoding IL-1, IL-6, IL-8, and TNF-α (2). We have recently shown that exposure of gastric epithelial cell lines to *H. pylori* potently activates NF-κB (15). Transcription factor induction by various *H. pylori* strains correlates with their ability to elicit IL-8 production. Indeed, cytokine production requires NF-κB activation, since its prevention by the antioxidant curcumin completely suppresses IL-8 production. Unlike other gram-negative bacteria, which induce NF-κB via their lipopolysaccharide molecules, *H. pylori* does not use lipopolysaccharide to activate the transcription factor (15). Rather, a gene located within the pathogenicity island, *cagE*, is required, since its mutation abolishes NF-κB induction. In this study we investigate the role of additional pathogenicity island genes in NF-κB activation. We show that while two genes, *cagF* and *cagN*, are not required for transcription factor activation, six genes, *cagE*, *cagG*, *cagH*, *cagI*, *cagL*, and *cagM*, are absolutely necessary, since isogenic *Helicobacter* strains carrying mutations in these loci no longer induce NF-κB activity. We propose that the proteins encoded by these genes form a surface structure which acts as the NF-κB-inducing agent.

Proteins encoded by the *cag* pathogenicity island are required for NF-κB activation. In order to investigate whether proteins encoded in the recently discovered *cag* pathogenicity island are required for NF-κB activation, KATO-III cells (ATCC HTB 103) were cocultured with various *Helicobacter* strains: a wild-type G27 strain as well as 12 isogenic strains, each with a mutation in a single gene encoded within the pathogenicity island (6). Coculture was performed as reported previously (15). Subsequently, the cells were harvested and total

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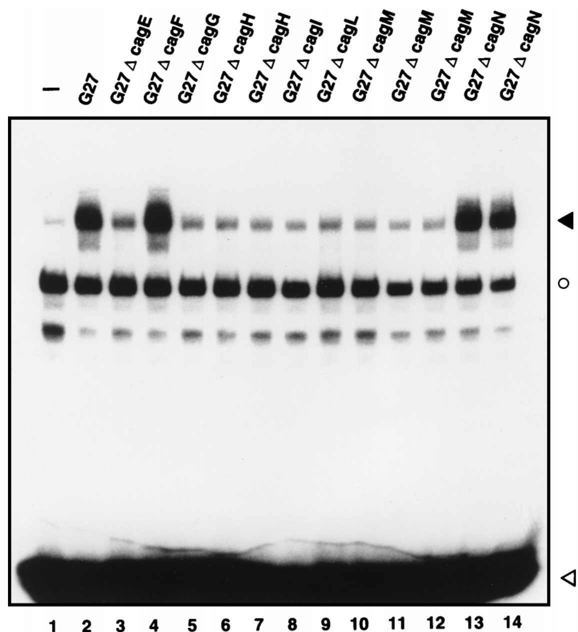

FIG. 1. Proteins encoded by the *cag* pathogenicity island are required for NF-κB activation. KATO-III cells were cocultured with 1 ml of bacterial culture of *H. pylori* G27 (lane 2) or its isogenic mutants (lanes 3 to 14). Control cells were left untreated (lane 1). After 1 h of coculture, total cell extracts were prepared and assayed by EMSA with a high-affinity κB-binding site as a probe. The closed arrowhead indicates specific NF-κB complexes. The open circle denotes nonspecific binding to the probe, and the open arrowhead indicates unbound oligonucleotide.

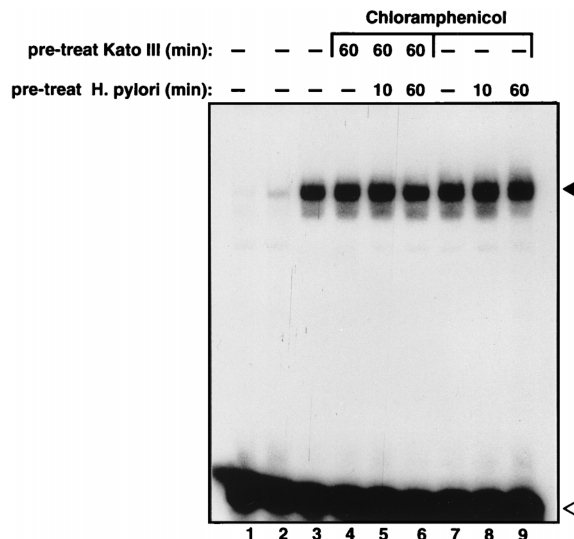

FIG. 2. The NF-κB-activating product is preformed prior to cell contact. KATO-III cells were either left untreated (lanes 1 to 3 and 7 to 9) or pretreated for 60 min with 2 mg of chloramphenicol per liter (lanes 4 to 6). Similarly, liquid cultures of *H. pylori* G27 were either left untreated (lanes 1 to 4 and 7) or pretreated for 10 min (lanes 5 and 8) or 60 min (lanes 6 and 9) with chloramphenicol. Subsequently, the epithelial cells and the bacteria were cocultured for 1 h, after which total cell extracts were prepared and assayed for NF-κB DNA binding by EMSA. The closed arrowhead indicates specific NF-κB complexes, and the open arrowhead indicates unbound oligonucleotide.

cell extracts were prepared as previously described (15, 19, 20). These were assayed for NF-κB DNA binding by an electrophoretic mobility shift assay (EMSA) as described previously (15). Coculture of KATO-III cells with the wild-type G27 strain induces a novel protein-DNA complex, which we have previously identified as NF-κB (Fig. 1, lane 2) (15). Strains with mutations in the genes encoding *cagF* and *cagN* also activate NF-κB (lanes 4, 13, and 14), while strains with mutations in six other genes, *cagE*, *cagG*, *cagH*, *cagI*, *cagL*, and *cagM*, no longer induce the transcription factor (lanes 3 and 5 to 12). We cannot exclude a polar effect in some of our mutants. Therefore, not all six Cag proteins may be required for NF-κB induction.

The NF-κB-activating product is preformed prior to cell contact. We wished to determine whether the NF-κB-inducing *H. pylori* proteins are preformed in the bacteria or become expressed only after contact with gastric epithelial cells. We therefore preincubated KATO-III cells with 2 mg of chloramphenicol per liter for 60 min. In addition, liquid *Helicobacter* cultures were also pretreated with 2 mg of chloramphenicol per liter for 10 or 60 min at 37°C, after which they were nonviable as shown by culture experiments. Pretreated or untreated KATO-III cells were cocultured with chloramphenicol-treated or untreated bacteria for 1 h. Total cell extracts were prepared and analyzed for NF-κB DNA binding (Fig. 2). Chloramphenicol treatment neither of the bacteria nor of the epithelial cells had any effect on the ability of *Helicobacter* to induce NF-κB activation. Therefore, the NF-κB-inducing factor is preformed in the bacterium before exposure to the epithelial cells.

We propose that proteins encoded in the *cag* pathogenicity island form a multimeric structure on the *H. pylori* surface. When bacteria are cocultured with gastric epithelial cells, this structure is capable of eliciting a signal transduction cascade which leads to activation of transcription factor NF-κB. Future research will aim at elucidating the molecular components of this signal transduction pathway. For example, it is not clear whether *H. pylori* uses a specific receptor on the epithelial cell surface. It has recently been suggested that tyrosine kinases are required for NF-κB activation by *H. pylori*; however, the enzymes involved remain unknown (22). Elucidation of this signal transduction pathway may point out novel therapeutic targets. Continuous and recurring NF-κB activation, which leads to production of IL-8 as well as other inflammatory cytokines such as IL-1, IL-6, and TNF-α, is a critical step in establishing the chronic inflammation seen in *H. pylori* gastritis. By inhibiting NF-κB activation, the secretion of inflammatory cytokines could be abolished. This inhibition may impede the establishment of a chronic *H. pylori* gastritis, perhaps rendering the infection asymptomatic.

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